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ARTICLE

The Bioavailable Octapeptide Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro Stimulates Nitric Oxide Synthesis in Vascular Endothelial Cells

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Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro (GAXGLXGP, X: Hyp), an octapeptide contained in chicken collagen hydrolysate, inhibits angiotensin I-converting enzyme activity in vitro. Intestinal Caco-2 and bovine aortic endothelial cells (BAECs) were used to investigate whether the transported GAXGLXGP improves vascular function. When GAXGLXGP was added to the apical side of Caco-2 monolayers, the intact form of GAXGLXGP was released to the basolateral side without incorporation into the cells. This transport was energy-independent but was associated with tight junction permeability. GAXGLXGP was then added to BAECs, and endothelial nitric oxide (NO) synthase (eNOS) activation was examined. GAXGLXGP at a concentration of 10 μM stimulated production of NO during a 1 h incubation. This event involved phosphorylation of eNOS at Ser^{1179} without a change in the total eNOS protein level. These findings indicate that GAXGLXGP absorbed intact through the intestinal epithelium has direct effects on eNOS activity in vascular endothelial cells, leading to NO synthesis, thereby suggesting the potential for improvement in vascular function.

KEYWORDS: Octapeptide; Caco-2 cells; bioavailability; BAECs; nitric oxide; eNOS

INTRODUCTION

During the past two decades, various food protein-derived peptides have gained widespread attention as physiologically active agents; these peptides have opioid (*1*), antibacterial (*2*), immunostimulating (*3*), antioxidant (*3*), and antihypertensive properties (*4*). Antihypertensive peptides have received much attention, because they are thought to be promising for use in patients with mild hypertension (*5*).

Previously, Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro (GAXGLXGP, X: Hyp), an octapeptide prepared from type I collagen from chicken legs, has been reported to show antihypertensive activity in vivo and in vitro (*6*, *7*). This octapeptide has strong angiotensin I-converting enzyme (ACE)-inhibitory effects in vitro (IC_{50} = 29.4 μM). In vitro assays have demonstrated that several peptides derived from food exert ACE-inhibitory activity (*8*). Most of them usually fail to be active when used in vivo, because the reported peptides are merely substrates of ACE or compete with the substrate in the enzyme assay (*9*). However, Saiga et al. found that a type I collagen peptide (Gly-Phe-Hyp-Gly-Thr-Hyp-Gly-Leu-Hyp-Gly-Phe) derived from chicken breast extract inhibited ACE activity and lowered blood pressure in spontaneously hypertensive rats (*10*, *11*). To express their antihypertensive effects in vivo, these peptides must be absorbed intact and transported across the intestinal epithelium to such target organs as the blood vessels.

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Endothelial cells line the internal surfaces of blood vessels and play critical roles in vascular biology, including in vasoconstriction and vasodilatation (*12*, *13*), blood clotting (*14*), and barrier function (*15*). Endothelial dysfunction is a hallmark of the development of vascular diseases such as atherosclerosis and hypertension. Endothelial nitric oxide (NO) synthase (eNOS) catalyzes the production of NO, which in turn mediates arteriolar vasodilatation, reduces peripheral resistance, and lowers the blood pressure (*16*, *17*). Thus, factors that elevate the function of this enzyme can lead to endothelial-dependent vasodilatation.

We hypothesized that a peptide that was absorbed intact and activated the microvascular eNOS could be a candidate bioavailable factor for use in vascular protection. Here, we investigated the bioavailability of the above-mentioned octapeptide by using the human colon carcinoma cell line Caco-2 and bovine aortic endothelial cells (BAECs). First, GAXGLXGP was added to Caco-2 cells to examine whether it was able to pass through the intestinal cells in the intact form. Then, GAXGLXGP was added to BAECs and examined for its ability to activate endothelial eNOS. This method is useful as an in vitro model for assessing vasoprotective compounds derived from foods.

MATERIALS AND METHODS

Materials. Caco-2 cells and BAECs were purchased from DS Pharma Biomedical (Osaka, Japan). Chemically synthesized and chromatographically purified GAXGLXGP was obtained from BEX Co. (Tokyo, Japan), and Val-Pro-Pro was obtained from Bachem (Bubendorf, Switzerland). Bradykinin (BK), 2-(*N*-morpholino)ethanesulfinic acid (MES), and *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES) were purchased from Sigma Chemical (St. Louis, MO). A Cell Counting Kit-8

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(CCK-8), sodium azide, cytochalasin B (Cyto B), and NO_2/NO_3 assay kit-FX were obtained from Wako Pure Chemical (Osaka, Japan). Other chemicals were commercial products of the highest grade available.

Cell Culture. Caco-2 cells were cultured in Eagle's minimum essential medium (DS Pharma Biomedical) supplemented with 10% fetal bovine serum (MP Biomedicals, Aurora, OH), 1% nonessential amino acids (MP Biomedicals), 2 mM L-glutamine (DS Pharma Biomedical), 50 units/mL penicillin (Gibco-BRL, Grand Island, NY), and 50 μg/mL streptomycin (Gibco-BRL). The cells were cultured in a monolayer under a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. After subculture to 80−90% confluence, they were harvested by trypsin treatment and then seeded on the apical side of a 12-well BD BioCoat Intestinal Epithelial Environment (1.0 μm pore, 10.5 mm diameter, and 0.9 cm^2/insert, BD Biosciences, San Jose, CA) at a density of 4.0×10^5 cells/well and then cultured per the manufacturer's instructions. The cells on the inserts were used to test the permeability of GAXGLXGP. Alternatively, Caco-2 cells were seeded on 96-well plates at a density of 1.0×10^4 cells/well; after incubation of the cells for 24 h, their viability was measured.

BAECs were cultured in CSC serum-containing medium supplemented with CultureBoost (Cell Systems, Kirkland, WA). BAECs were seeded on 24-well plates or on 6.0 cm dishes coated with collagen type I and cultured at 37 °C under 5% CO_2. The cells on the plates were used for NO measurement and those in the dishes for immunoblot analysis. These experiments were conducted on BAECs before their fifth passage.

Incubation of Caco-2 Monolayers with GAXGLXGP. For the permeation studies, Caco-2 cells at passages 49−55 were used for experiments 3 or 4 days after seeding. The transepithelial electrical resistance (TEER) of the Caco-2 monolayers, which was measured with a Millicell-ERS (Millipore, Bedford, MA) (*18*), was approximately 600 Ω cm^2. The inserts were washed twice with Hanks' balanced salt solution (HBSS), placed in a CO_2 incubator at 37 °C for 30 min, and used in the permeability experiments. GAXGLXGP was diluted with HBSS (pH adjusted to 6.0 with MES) to four concentrations from 10 μM to 1.0 mM. Each diluted sample (500 μL) was added to the apical side of the Caco-2 monolayers; the basolateral side contained 1500 μL of HBSS (pH adjusted to 7.4 with HEPES). Following incubation at 37 °C for an appropriate time, the apical and basolateral solutions were collected.

After the transport study, the Caco-2 monolayers were washed with ice-cold phosphate-buffered saline (PBS) and then trypsinized to suspend the cells. The suspensions were further washed three times with ice-cold PBS and then homogenized by ultrasonication. The homogenate was centrifuged at 15000*g* for 30 min, and the recovered supernatants were used as cellular extracts.

All of the experiments were conducted in triplicate. GAXGLXGP concentration in each was determined by HPLC. A permeability coefficient (P_{app}) for GAXGLXGP was calculated according to the method of Walgren et al. (*19*).

GAXGLXGP Transport Route in Caco-2 Monolayers. To identify the transport routes involved in the transepithelial transport of GAXGLXGP, Caco-2 monolayers were exposed to sodium azide (an ATP synthesis inhibitor, 10 mM) and Cyto B (an accelerant of the paracellular transport, 5.0 μg/mL) for 30 min before the transport experiment. GAXGLXGP at 1.0 mM was added to the apical side of Caco-2 monolayers. After incubation for 2 h, the amounts of GAXGLXGP in the apical, intracellular, and basolateral phases were determined by HPLC.

Cell Viability. To assay the cytotoxicity of GAXGLXGP, Caco-2 cells on 96-well plates were incubated with GAXGLXGP at concentrations of 10, 100, and 500 μM and 1.0 mM. CCK-8 was employed to evaluate cell viability. Briefly, 10 μL of CCK-8 solution was added to the wells, and the samples were then incubated at 37 °C for 2 h. After incubation, the difference in absorbance at a wavelength of 450/620 nm was measured with a microplate reader (TECAN Trading AG, Männedorf, Switzerland).

Quantification of GAXGLXGP by HPLC. Transported GAXGLXGP was detected by a Shimadzu HPLC system equipped with LCsolution software, a LC-10AD*vp* pump, a SIL-10AD*vp* autosampler, a CTO-10A*vp* column oven, and an SPD-10A*vp* UV−vis detectior (Shimadzu, Kyoto, Japan) to monitor wavelengths at 214 and 280 nm. The column, an Inertsil ODS-3 (250 × 4.6 mm i.d., S-5 μm, GL Sciences, Tokyo, Japan), was maintained at 40 °C. Gradient elution was performed with solution A, composed of distilled water including 0.1% trifluoroacetic acid (TFA), and with solution B, comprising 80% acetonitrile in water in the presence of 0.1% TFA. The process began with 0% B and increased to 50% B (15−30 min); B was held at 50% (30−45 min) and then decreased to 0% (45 to 60 min). The flow rate was 1.0 mL/min.

The apical, intracellular, and basolateral solutions mentioned above were dried in a centrifugal concentrator (CC-105, TOMY, Tokyo, Japan). The dried materials were dissolved in HPLC solution A and used for the analyses after being filtered through a 0.5 μm DISMIC-3JP filter (ADVANTEC, Tokyo, Japan). Calibration curves for GAXGLXGP were constructed with concentrations of 1.0−100 μM.

Measurement of NO Production. We determined NO production by measuring the sum concentration of NO_2^- and NO_3^- in culture supernatants using a fluorometric assay kit in accordance with the manufacturer's instructions. BAECs were grown in 24-well plates and serum starved for 24 h. The cells were then incubated with either 10 μM GAXGLXGP or 1.0 μM BK for an appropriate time. Culture medium was collected for NO production assay. Fluorescence was measured in a fluorescence microplate reader (Perkin-Elmer, Turku, Finland) with excitation and emission wavelengths of 360 and 460 nm, respectively. Fluorescence data were converted into concentrations based on standard curves constructed with $NaNO_3$ (0.16−10 μmol/L).

Immunoblot Analysis. To characterize the effects of GAXGLXGP, BAECs were cultured on 6.0 cm dishes and serum starved for 24 h. The cells were then incubated with various concentrations of GAXGLXGP or 1.0 μM BK for 10 min or with 10 μM GAXGLXGP for an appropriate time. Reactions were terminated by aspiration, and the cells were washed twice with ice-cold PBS. They were lysed with lysis buffer supplemented with protease and phosphatase inhibitors and then subjected to centrifugation at 15000*g* for 20 min at 4 °C. The supernatant was then boiled with Laemmli sample buffer (Bio-Rad Laboratories, Hercules, CA) for 5 min and loaded in equal amounts of total protein on 10% SDS-PAGE gels. The separated proteins were transferred onto polyvinylidene difluoride membranes (Bio-Rad Laboratories) and probed with antibody to phospho-eNOS (Ser^{1179}), followed by secondary antibody conjugated with horseradish peroxidase. The immunoreactive proteins were detected by using ECL chemiluminescence kits (GE Healthcare, Buckinghamshire, U.K.). Membranes were stripped and reprobed with eNOS antibody to monitor for equal protein loading. Quantitative analysis of band intensity was done with Quantity One-4.6.2 software (Bio-Rad Laboratories). The activated protein (phospho-eNOS) level was normalized to the eNOS level from the same sample. Sources of the primary antibodies used here were as follows: phospho-eNOS (Ser^{1179}), Cell Signaling Technology (Beverly, MA); and eNOS, Sigma Chemical. The secondary antibody was anti-rabbit IgG (Millipore, Bedford, MA).

Statistical Analysis. Data were analyzed by one-way ANOVA followed by Fisher's PLSD post hoc test with Stat View software (Abacus Concepts, Berkeley, CA). Probability values of < 0.05 were considered to be statistically significant.

RESULTS

Transepithelial Transport of GAXGLXGP across Caco-2 Monolayers. A cytotoxicity test using CCK-8 showed that GAXGLXGP (≤1.0 mM) had no toxic effects on Caco-2 cells after 2 h of incubation (data not shown). GAXGLXGP at a concentration of 1.0 mM was then added to the apical side of Caco-2 monolayers, and after incubation of the monolayers at 37 °C for 0.5−2 h, levels of GAXGLXGP in the basolateral solutions were determined by HPLC. Under the chromatographic conditions used in this experiment, the retention time for GAXGLXGP was 29.8 min. **Figure 1A** shows that the amount of GAXGLXGP transported transepithelially to the basolateral side increased linearly up to 2 h.

The effect of the concentration of GAXGLXGP on its transepithelial flux was examined. The flux of GAXGLXGP was directly proportional to the concentration of the peptide between 10 μM and 1.0 mM (**Figure 1B**). Transepithelial transport of GAXGLXGP was therefore evaluated in the following experiments by measuring the apical, intracellular, and basolateral concentrations after incubation of the monolayers with 1.0 mM GAXGLXGP for 2 h.

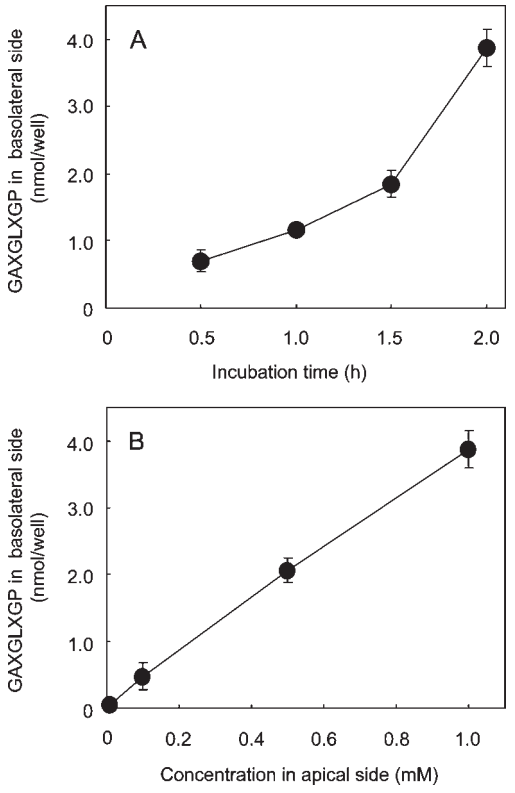

Figure 1. Time and dose dependency of GAXGLXGP transport across Caco-2 monolayers. (**A**) GAXGLXGP (1.0 mM) was added to the apical side. After incubation at 37 °C for the times shown, accumulation of GAXGLXGP in the basolateral solution was monitored. (**B**) GAXGLXGP at the concentrations shown was added to the apical side. After incubation at 37 °C for 2 h, accumulation of GAXGLXGP in the basolateral solution was monitored. Values are means ± SD with $n = 3$.

Table 1. Transport of GAXGLXGP across Caco-2 Cell Monolayers

treatment	P_{app}[a] (cm/s × 10^7)
Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro	1.99 ± 0.14
Val-Pro-Pro	2.09 ± 0.47

[a] P_{app} from the apical to basolateral phases was calculated from the results of GAXGLXGP treatment for 2 h or from the data from Val-Pro-Pro treatment for 2 h, as mentioned under Materials and Methods. Values are means ± SD with $n = 3$.

The P_{app} value of GAXGLXGP from the apical to the basolateral side was 1.99 ± 0.14 cm/s × 10^7 (**Table 1**). This was slightly lower than that of the ACE-inhibitory tripeptide Val-Pro-Pro (2.09 ± 0.47 cm/s × 10^7), but the difference was not significant, suggesting that the bioavailabilities of the two peptides were comparable.

Resistance to Hydrolysis of GAXGLXGP during Transport of Caco-2 Monolayers. GAXGLXGP at 1.0 mM was added to the apical chamber of Caco-2 monolayers, and the concentrations of intact GAXGLXGP and their hydrolysates (free amino acids and decomposed peptides) in the Caco-2 cells, as well as in the apical/basolateral solutions, were determined. The distribution of GAXGLXGP after the 2 h transport experiment is summarized in **Table 2**. About 0.8% of GAXGLXGP disappeared from the apical side and appeared in the basolateral side as an intact form during the 2 h incubation. Comparison of the amount of intracellular GAXGLXGP with that in untreated cellular extracts revealed no change on the HPLC chromatograph. Free amino acids and decomposed peptides were not detectable in the apical and basolateral solutions. These results indicated that GAXGLXGP was released to the basolateral side as an intact form without incorporation into the Caco-2 cells.

Table 2. Distribution of GAXGLXGP in the Caco-2 Transport Experiment[a]

	amount (nmol/well)	% of total
apical solution	496.26 ± 0.33	99.2
cellular extract	nd	0
basolateral solution	3.74 ± 0.30	0.8
total GAXGLXGP	500	100

[a] Five hundred microliters of a GAXGLXGP solution (1.0 mM) was added to the apical side of Caco-2 cell monolayers. After 2 h of incubation, GAXGLXGP contents in the apical and basolateral solutions and the cellular extract were measured. Values are means ± SD with $n = 3$. nd, not detected.

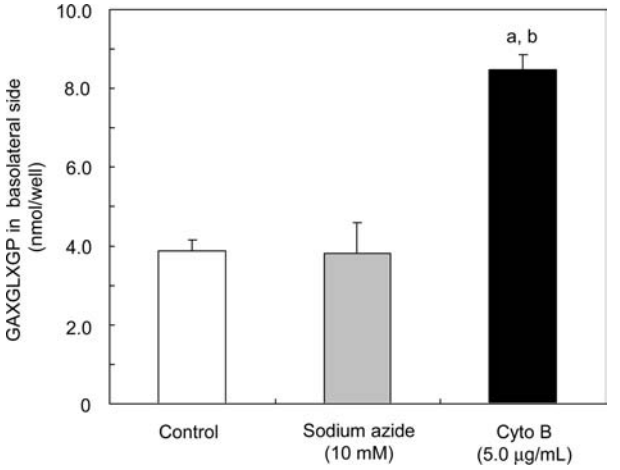

Figure 2. Effects of sodium azide and Cyto B on the transepithelial transport of GAXGLXGP across Caco-2 monolayers. GAXGLXGP (1.0 mM) was added to the apical side in the presence or absence of sodium azide or CytoB. The concentration of GAXGLXGP in the basolateral solution was measured after 2 h of incubation. Values are means ± SD with $n = 3$. The letter "a" indicates significant difference from the control ($P < 0.001$), and "b" indicates significant difference from sodium azide treated Caco-2 cells ($P < 0.001$).

Contribution of Paracellular Diffusion to Transepithelial Flux of GAXGLXGP. To investigate whether or not energy-dependent transport by transporters or transcytosis participated in permeation of GAXGLXGP, sodium azide was added to the apical side 30 min before the transport experiment (**Figure 2**). Preincubation with sodium azide did not affect the transport rate, suggesting that transport of GAXGLXGP across the Caco-2 monolayers was energy-independent. The integrity and transport function of the Caco-2 monolayers were confirmed by using Gly-Pro, a good substrate for peptide transporter (*20*) (data not shown). Paracellular diffusion is thought to be an important pathway for such hydrophilic nutrients as oligopeptides and minerals (*21*, *22*). Thus, the effect of Cyto B, a tight junction (TJ) modulator, on apical to basolateral transport was also examined. Cyto B is known to increase paracellular passive diffusion across Caco-2 monolayers by regulating myosin light chain kinase, leading to the modulation of TJ (*23*). After Cyto B caused a loss of the epithelial TJ (TEER value decreased from approximately 600 to 150 Ω cm^2), the rate of transport of GAXGLXGP across the Caco-2 monolayers increased dramatically (**Figure 2**). About 1.6% of GAXGLXGP disappeared from the apical side after preincubation with Cyto B and appeared in the basolateral side in an intact form. The P_{app} for GAXGLXGP was accelerated to 4.36 ± 0.20 cm/s × 10^7. These results indicate that paracellular transport plays a major role in transepithelial transport of GAXGLXGP.

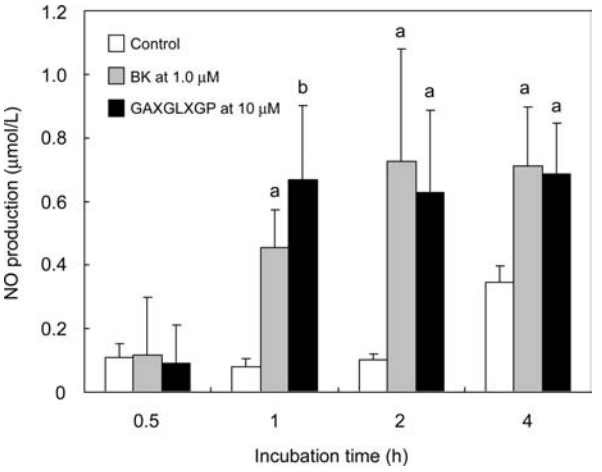

Figure 3. GAXGLXGP stimulates NO production in BAECs. Serum-deprived BAECs were incubated with 1.0 μM BK or with 10 μM GAXGLXGP for the times shown. NO production in culture supernatants was measured as described under Materials and Methods. Values are means $\pm$ SD with $n = 3$. Letters indicate significant difference from control at each incubation time (a, $P < 0.05$; b, $P < 0.01$).

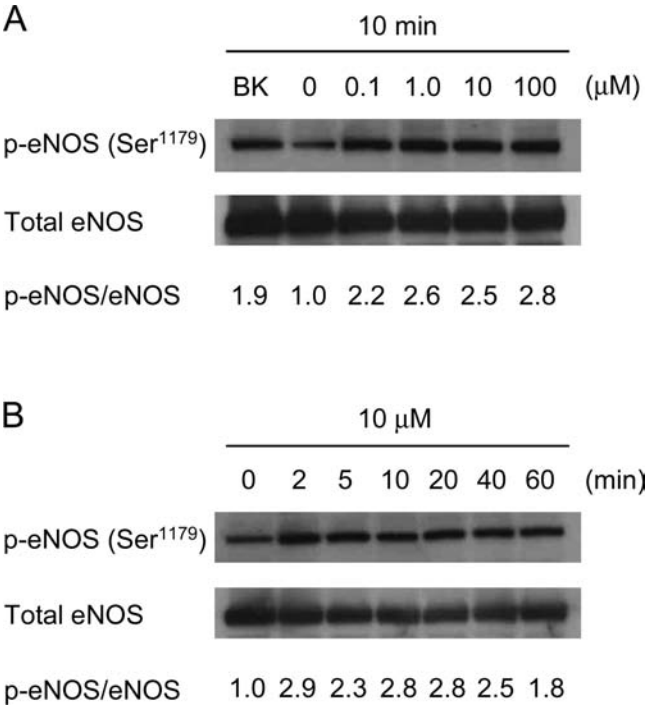

Figure 4. Effect of GAXGLXGP on eNOS activation. Serum-deprived BAECs were incubated for 10 min with GAXGLXGP at the concentrations shown (**A**) or with 10 μM GAXGLXGP for the indicated times (**B**). Western blot analysis was performed to detect phosphorylation of eNOS at Ser^{1179} (bovine sequence) and total eNOS. Equal amounts of protein in whole-cell extracts were separated and electrophoretically blotted as described under Materials and Methods.

GAXGLXGP Enhances NO Production in BAECs. Endothelium-derived NO is an important physiological vasodilator (*16*, *17*). We examined whether GAXGLXGP treatment could directly stimulate production of NO from the vascular endothelium in BAECs. The levels of NO production during 30 min of incubation with GAXGLXGP and BK were low and difficult to differentiate from the control level. GAXGLXGP at a concentration of 10 μM, however, significantly stimulated NO production following 1 h of incubation compared with that of control cells (**Figure 3**). The time course experiment showed that NO production induced by 10 μM GAXGLXGP had dramatically increased by the end of 1 h of exposure, to 780% of that at 30 min of incubation. Thus, stimulation of NO production was considered to begin before 1 h after treatment. The similarity in the levels of NO production at 2 and 4 h after treatment of cells with equipotent doses of GAXGLXGP or BK (1.0 μM) confirmed the high potencies of GAXGLXGP in causing vasodilation.

GAXGLXGP Stimulates Phosphorylation of eNOS in BAECs. BAECs on 6.0 cm dishes were incubated with GAXGLXGP at various concentrations for 10 min or with 10 μM GAXGLXGP for various times. These conditions were employed on the basis of the levels and minimum detection time of food-derived collagen peptides in human blood after ingestion of gelatin hydrolysates (*24*). Because eNOS is activated by direct enzyme phosphorylation without an increase in protein expression, we examined whether GAXGLXGP rapidly induced eNOS phosphorylation. Immunoblots showed that GAXGLXGP at even the lowest concentration (0.1 μM) stimulated an increase in the phosphorylation of eNOS (Ser^{1179}) within 10 min of incubation. BK-stimulated cells used as positive controls showed a definite increase in the phosphorylation of eNOS at Ser^{1179}. Maximum stimulation (280% of that in the control) occurred at 100 μM GAXGLXGP (**Figure 4A**). At 10 μM, the effect of GAXGLXGP on eNOS phosphorylation peaked at 2 min of incubation and decreased thereafter, but was maintained through 1 h of incubation (**Figure 4B**). Expression of total eNOS protein remained almost unchanged in these studies.

DISCUSSION

These results indicated that GAXGLXGP, an octapeptide contained in chicken collagen hydrolysate, was a factor that was bioavailable for use in vascular protection. A number of food-derived peptides have been reported to have biologically active properties (*25*), with milk proteins being the most commonly known source (*26*). Among the bioactive peptides, antihypertensive peptides—particularly ACE-inhibitory peptides—have received considerable attention (*5*). The ACE-inhibitory peptides derived from vertebrate muscle protein have shown IC_{50} values ranging from 0.21 to >1000 μM (*27*). In comparison, GAXGLXGP has shown a strong ACE-inhibitory effect (IC_{50} = 29.4 μM) (*7*). An important point, however, is whether or not these peptides are bioavailable.

To determine whether GAXGLXGP was able to pass through intestinal cells, we used Caco-2 human intestinal epithelial cells, which have a variety of functions in the small intestine and have been used as a model of the intestinal epithelium (*18*). When GAXGLXGP was added to Caco-2 monolayers with incubation for 2 h, the P_{app} value from the apical to the basolateral side was 1.99 ± 0.14 cm/s $\times 10^7$, of a magnitude similar to that in Val-Pro-Pro (**Table 1**). Nakamura et al. have reported that the IC_{50} of Val-Pro-Pro for ACE was 9.0 μM (*4*). Seppo et al. suggested that normal daily use of fermented milk containing Val-Pro-Pro had a blood pressure-lowering effect in hypertensive subjects (*28*). Because both parameters (P_{app} value and IC_{50} for ACE) for GAXGLXGP were comparable with those for Val-Pro-Pro, GAXGLXGP was anticipated to be biologically active. Quirós et al. found that antihypertensive Leu-His-Leu-Pro-Leu-Pro, a hexapeptide contained in fermented milk, was hydrolyzed into a pentapeptide, His-Leu-Pro-Leu-Pro, before transport across the intestinal epithelium (*29*). Interestingly, our results in vitro indicated that GAXGLXGP was resistant to cellular peptidase and was detectable in only the intact form in the basolateral phase (**Table 2**). Additionally, our previous data suggested that

GAXGLXGP was not digested by gastrointestinal proteases such as pepsin and a mixture of trypsin and α-chymotrypsin (*6*). Thus, it is likely that a part of the ingested GAXGLXGP is absorbed without modification and transported to the target organs.

There are three possible mechanisms for the intestinal transport of oligopeptides. One is paracellular transport through intracellular junctions, and the others are transporter (PepT1)-mediated transport and transcytosis. Paracellular transport is an energy-independent mechanism for absorption of water-soluble low molecular weight substances, including minerals and oligopeptides (*21*, *22*). Of the other mechanisms, PepT1 mediates the transport of di- and tripeptides (*30*), and transcytosis has been reported to transport certain peptides (*31*). Sun et al. found that paracellular transport was the main mechanism of transport of the intact ACE-inhibitory hexapeptide Lys-Val-Leu-Pro-Val-Pro (*32*). Furthermore, paracellular transport, but not PepT1-mediated transport, is the major flux mechanism for the transport of intact Val-Pro-Pro across Caco-2 monolayers (*33*). To determine whether paracellular transport was involved in the transepithelial transport of GAXGLXGP, we used a TJ modulator, CytoB (**Figure 2**). Acceleration of paracellular transport dramatically promoted the transport of GAXGLXGP. CytoB treatment of Caco-2 monolayers decreased their TEER value by 75%. The decrease in TEER value was accompanied by a doubling of the permeability of the monolayer to GAXGLXGP (**Figure 2**). These results indicated that transport of intact GAXGLXGP occurred mainly by paracellular diffusion.

Chicken collagen hydrolysate, which includes GAXGLXGP used in this study, can repair endothelial progenitor cells (EPCs) in impaired blood vessels (*34*). EPCs are recruited from the bone marrow, circulate in the blood, and play an important role in repairing endothelial cells at damaged sites in tissues (*35*). This prompted us to further investigate the direct effect of GAXGLXGP on vascular endothelial cells and potential mechanisms for improving vascular health. We focused our attention on eNOS as a possible signaling target for GAXGLXGP, because this enzyme is the major source of NO production in endothelial cells and is an important regulator of vascular homeostasis. NO plays a crucial role in blood vessel tone and hence blood pressure regulation (*16*, *17*). Our results in the BAECs demonstrated that GAXGLXGP was equal to BK in terms of NO production and eNOS phosphorylation (**Figures 3** and **4**). The activity of eNOS is regulated by phosphorylation at multiple sites. The activation site Ser^{1177} (in humans) or Ser^{1179} (in cattle) is phosphorylated by several protein kinases, including phosphatidylinositol 3-OH kinase/Akt, protein kinase A, and AMP-activated protein kinase, in response to various types of stimulation (*36*). In the future, it will be necessary to investigate the mechanisms of eNOS phosphorylation by GAXGLXGP. A major finding of our study is that the stimulation of NO production induced by GAXGLXGP occurred within 1 h after the start of treatment (**Figure 3**). Iwai et al. have reported that, after ingestion of gelatin hydrolysates, the levels of several food-derived collagen peptides in human blood plasma peak at 20−150 nmol/mL after 1 or 2 h and decrease to half the peak level by 4 h (*24*). Thus, part of the intact GAXGLXGP is probably circulated around the body at a micromolar level and mediates the arteriolar vasodilatation.

The mechanisms of action of chicken collagen hydrolysate in hypertension are not fully understood. We previously found that intake for 3 weeks of a beverage containing 2.9 g of chicken collagen hydrolysate with >6.2 mg of GAXGLXGP reduced blood pressure in mildly hypertensive subjects (*37*). On the basis of the estimation from the present results, around 50 μg of GAXGLXGP may get into the circulation and act as an antihypertensive peptide. On the other hand, a single dose of GAXGLXGP at 4.5 mg/kg of body weight lowered the systolic blood pressure in an animal experiment (*6*). Our current data demonstrated that GAXGLXGP significantly increased NO production in endothelial cells. Thus, our previous results that chicken collagen hydrolysate has antihypertensive effects can be explained, at least in part, by the stimulation of eNOS by GAXGLXGP. These findings potentially elucidate the basic mechanisms underlying the physiological effects of chicken collagen hydrolysate in the vasculature. Therefore, GAXGLXGP, which may be absorbed intact and activates microvascular eNOS, may be a candidate bioavailable factor for use in vascular protection.

ABBREVIATIONS USED

GAXGLXGP, Gly-Ala-Hyp-Gly-Leu-Hyp-Gly-Pro; BAECs, bovine aortic endothelial cells; NO, nitric oxide; eNOS, endothelial NO synthase; ACE, angiotensin I-converting enzyme; BK, bradykinin; MES, 2-(*N*-morpholino)ethanesulfinic acid; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); CCK-8, Cell Counting Kit-8; Cyto B, cytochalasin B; TEER, transepithelial electrical resistance; HBSS, Hanks' balanced salt solution; PBS, phosphate-buffered saline; P_{app}, permeability coefficient; TFA, trifluoroacetic acid; TJ, tight junction; EPCs, endothelial progenitor cells.

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